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The *TRK-T1* fusion protein induces neoplastic transformation of thyroid epithelium

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Genetic analysis of human papillary thyroid carcinomas (PTC) has revealed unique chromosomal translocations that form oncogenic fusion proteins and promote thyroid tumorigenesis in up to 60% of tumors examined. Although, the majority of thyroid specific translocations involve the growth factor receptor *c-RET*, variant rearrangements of the receptor for nerve growth factor, *NTRK1* have also been described. One such translocation, *TRK-T1*, forms a fusion protein composed of the carboxyl terminal tyrosine kinase domain of NTRK1 and the amino terminal portion of TPR (Translocated Promoter Region). To determine if *TRK-T1* expression can cause thyroid cancer *in vivo*, we developed transgenic mice that express the human TRK-T1 fusion protein in the thyroid. Immunohistochemical analysis of *TRK-T1* transgenic mouse thyroids revealed *TRK-T1* staining within the thyroid follicular epithelium. In contrast to nontransgenic littermates, 54% of transgenic mice developed thyroid abnormalities that included follicular hyperplasia and papillary carcinoma. Furthermore, all transgenic mice examined greater than 7 months of age developed thyroid hyperplasia and/or carcinoma. These data support the conclusion that *TRK-T1* is oncogenic *in vivo* and contributes to the neoplastic transformation of the thyroid. *Oncogene* (2000) **19,** 5729–5735.

Keywords: neoplasia; nerve growth factor receptor; papillary thyroid carcinoma; RET/PTC; transgenic mice; TRK-T1

Introduction

Genetic analysis of human papillary thyroid carcinomas has revealed common chromosomal translocations involving the growth factor receptors, *NTRK1* (*TRK-A*) and *c-RET*, that fuse the carboxyl terminal tyrosine kinase domain of the receptor to the amino terminal region of a ubiquitously expressed partner (Bongarzone *et al*., 1989). The *TRK* family of oncogenes result from a genetic fusion of the 3′ end of *NTRK1* to the 5′ end of different partner genes (Pierotti *et al*., 1996). The *TRK* oncogene results from a paracentric inversion of chromosome 1 that joins the 5′ end of *Tropomyosin* (*TPM3*) to *NTRK1* (Bongarzone *et al*., 1989; Martin-Zanca *et al*., 1986). Similarly, *TRK-T1* results from a paracentric inversion of chromosome 1 that joins the 5′ end of *TPR* to the 3′ end of *NTRK1* (Greco *et al*., 1992). Different rearrangements involving the same two genes, *TPR* and *NTRK1*, form the family members, *TRK-T2* and *TRK-T4* (Greco *et al*., 1992). Lastly, *TRK-T3* results from a translocation of chromosomes 1 and 3 that fuses the genes, *TFG* (*TRK* fused gene) and *NTRK1* (Greco *et al*., 1998, 1995). Likewise, the predominant family of PTC-specific fusion genes involves translocation of *c-RET* and form fusion proteins called RET/PTC (Pierotti *et al*., 1996).

RET/PTC fusion proteins are expressed in 60% of PTC (Rabes and Klugbauer, 1998), whereas *NTRK1*-related translocations are found less frequently, with a reported incidence of 0–12% in patients with PTC (Beimfohr *et al*., 1999; Bongarzone *et al*., 1998b; Delvincourt *et al*., 1996; Wajjwalku *et al*., 1992). Although the etiology of fusion gene formation is not clear, their appearance in patients with prior exposure to ionizing radiation is notable. For instance, recent studies of children with radiation-induced thyroid tumors from Belarus have found *RET/PTC* rearrangements in 78% of the PTC (Klugbauer *et al*., 1995, 1996). In contrast, *NTRK1* translocations were identified in 7% of the 81 Belarussion papillary thyroid carcinomas which were devoid of *RET* rearrangements (Beimfohr *et al*., 1999). Thus, though translocations of both *c-RET* and *NTRK1* are found in radiation-induced PTC, rearrangements of *c-RET* predominate. Nevertheless, these studies demonstrate an association between prior ionizing radiation exposure, the presence of translocations involving *NTRK1* and *c-RET* and the occurrence of papillary thyroid carcinoma.

Although the NTRK1 protein is known to function as a receptor for nerve growth factor, little is known about its pathological role in the genesis of thyroid carcinoma. The *NTRK1* proto-oncogene has been found rearranged in human papillary thyroid carcinoma, suggesting that NTRK1 function may be important for cancer development in a manner analogous to RET/PTC (Beimfohr *et al*., 1999; Bongarzone *et al*., 1996, 1989, 1998b; Greco *et al*., 1992; Sozzi *et al*., 1992; Wajjwalku *et al*., 1992). *In vitro*, the *TRK-T1* oncogene transforms NIH3T3 cells (Greco *et al*., 1992). Other *NTRK1* translocations transform NIH3T3 including *TRK*, *TRK-T2* and *TRK-T3* (Greco *et al*., 1992, 1995; Martin-Zanca *et al*., 1986). However, when *TRK-T1*

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was transfected into the rat epithelial thyroid cell line PC C13, it was unable to promote growth in soft agar or nude mice (Portella *et al*., 1999). In addition, PC C13 cells transfected with the oncogene *TRK-T1* retained a differentiated phenotype that included thyroglobulin expression and a dependency on growth factor supplementation, although iodide uptake was abrogated (Portella *et al*., 1999). These studies have shown that while *NTRK1* translocations are able to transform NIH3T3 cells, they are unable to change the growth or differentiation state of normal thyroid cells *in vitro*.

In order to study the *in vivo* function of thyroid specific oncogenes in the transformation of thyroid follicular epithelium, transgenic mouse strains were developed (Jhiang *et al*., 1996; Powell *et al*., 1998; Santoro *et al*., 1996). For example, two transgenic mouse strains engineered to express the human *RET/PTC1* fusion gene in the thyroid develop follicular hyperplasia and carcinoma (Jhiang *et al*., 1996; Santoro *et al*., 1996). Cytological features characteristics of human PTC, such as nuclear grooves and ground-glass nuclei were present, however, lymph node metastasis and local invasion were not observed and colloid production was variable between the two strains. These findings possibly reflect differences in transgene expression level, construct structure, or strain variability. An independent transgenic mouse strain expressing the human *RET/PTC3* oncogene, frequently develops solid-type thyroid carcinomas pathologically similar to the radiation-induced carcinomas observed in humans (Powell *et al*., 1998). Although cytological features such as ground-glass nuclei and nuclear grooves were not observed in *RET/PTC3* transgenic mice, other features common to the human disease including papillary structures, infrequent metastasis to regional lymph nodes and solid sheets of tumor cells were found (Powell *et al*., 1998). Tumor development in *RET/PTC* transgenic mice provides support for the theory that thyroid-specific fusion proteins can initiate malignant transformation of thyroid follicular epithelium.

Although the neoplastic role of *RET/PTC* family members has been established *in vivo*, translocations involving *NTRK1* have not been studied. The relatively rare occurrence of *NTRK1* translocations found in PTC, the inability of *TRK-T1* to fully transform PC C13 cells *in vitro*, and the lack of a mouse strain expressing an *NTRK1* fusion protein, led us to question the ability of the *NTRK1* protooncogene to initiate cancer in thyroid follicular epithelium. Accordingly, we have generated transgenic mice encoding the human *TRK-T1* transgene expressed in the murine thyroid under the control of the bovine thyroglobulin promoter. The development of differentiated carcinomas in these mice supports a direct role for *TRK-T1* in the development of thyroid cancer. Furthermore, *TRK-T1* transgenics will be a valuable animal model to study the function of this variant thyroid oncogene.

Results

Generation and analysis of TRK-T1 transgenic mice

Thyroid-specific expression of the *TRK-T1* fusion gene was obtained using a minigene construct containing 2 kb of the bovine thyroglobulin promoter (−2036 to +9) proximal to 0.7 kb of the human β-globin intron, the complete 1.7 kb coding sequence of the human *TRK-T1* fusion gene and an SV40 polyadenylation signal sequence (Figure 1). High-level expression of the *TRK-T1* fusion protein was observed in transfected PC C13 rat thyroid cells, confirming the functionality of this construct (Figure 2). To develop a transgenic mouse line, B6C3F1 zygotes were microinjected and embryos were transferred to pseudopregnant females. Progeny were genotyped using PCR and Southern blotting techniques as previously described (Powell *et al*., 1998). Three independent founder lines of *TRK-T1* transgenic mice were generated: 5215, 5218 and 5221. Transgene expression and tissue specificity was verified by RNase protection analysis of total RNA isolated from mouse thyroids (Figure 3). All three lines expressed the *TRK-T1* oncogene at similar levels when compared to a *β-Actin* internal control.

Immunohistochemical analysis of thyroid-specific protein expression in TRK-T1 transgenic thyroids

TRK-T1 thyroids were examined using immunohistochemical techniques with an antibody specific for the carboxyl terminus of NTRK1. NTRK1 specific staining was observed in follicular epithelial cells of transgenic, but not nontransgenic thyroids (Figure 4 and data not shown). No quantitative differences in *TRK-T1* mRNA (Figure 3) or protein (data not shown) expression were observed between the transgenic lines 5215, 5218 and 5221. *TRK-T1* transgenic thyroids retained a differentiated phenotype as indicated by the staining of colloid with an antibody specific for thyroglobulin (Figure 5a). *TRK-T1* transgenic thyroids expressed the thyroid differentiation genes thyroglobulin, thyroid peroxidase and thyroid stimulating hormone receptor at levels similar to *RET/PTC3* transgenic mice as measured by RT–PCR (Figure 6).

TRK-T1 transgenic mice develop thyroid hyperplasia and carcinoma

In order to evaluate the pathological changes associated with TRK-T1 expression, we sacrificed transgenic mice at various ages between 2 and 24 months old. *TRK-T1* transgenic mice, line 5215, were examined

Figure 1 Schematic of the construct used to generate the *TRK-T1* transgenic mice. BTG-Pro, bovine thyroglobulin promoter (2 kb); β-globin intron (0.7 kb); *TPR*, translocated promoter region, the 5′ *TRK-T1* fusion partner (588 bp); *NTRK1*, the 3′ *TRK-T1* fusion partner (1158 bp); pA, SV40 polyadenylation signal (1 kb)

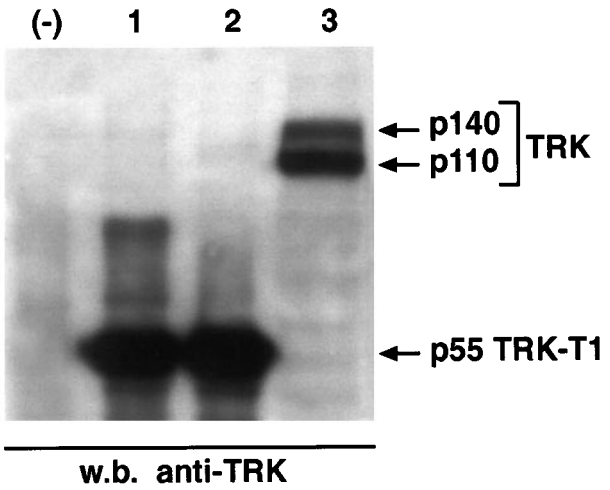

Figure 2 Western blot analysis of NTRK1 protein expression in *TRK-T1* transfected PC C13 cell line. PC C13 cells were co-transfected with either 10 μg *TRK-T1* transgenic DNA construct shown in Figure 1 and 1 μg of *pSV2-neo* or the positive control *NTRK1* plasmid *pDM-69*. Three days after transfection, thyroid cultures were lysed and proteins resolved on a 10% polyacrylamide gel. Following transfer onto nylon and staining using an antibody against NTRK1, expression of the NTRK1 protein was observed in *TRK-T1* transfectants (lanes 1 and 2) and in the NTRK1 transfectant positive control (lane 3) but not the negative control (−). Lane (−): untransfected cells; lanes 1, 2: two independent *TRK-T1* transfectants; lane 3: *pDM-69*, *NTRK1* transfectant (p140 and p110, fully and partially glycosylated forms)

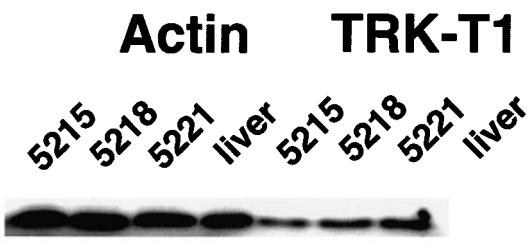

Figure 3 RNAse protection analysis of the three *TRK-T1* transgenic mouse lines, 5215, 5218 and 5221. Total RNA was extracted from the thyroids of the three transgenic mouse lines or mouse liver and hybridized to a radiolabeled RNA probe from either the *TRK-T1* oncogene (180 bp probe) or *β-Actin* (330 bp probe) and then RNAse digested. Protected fragments were resolved on a 5% denaturing polyacrylamide gel and visualized by audioradiography

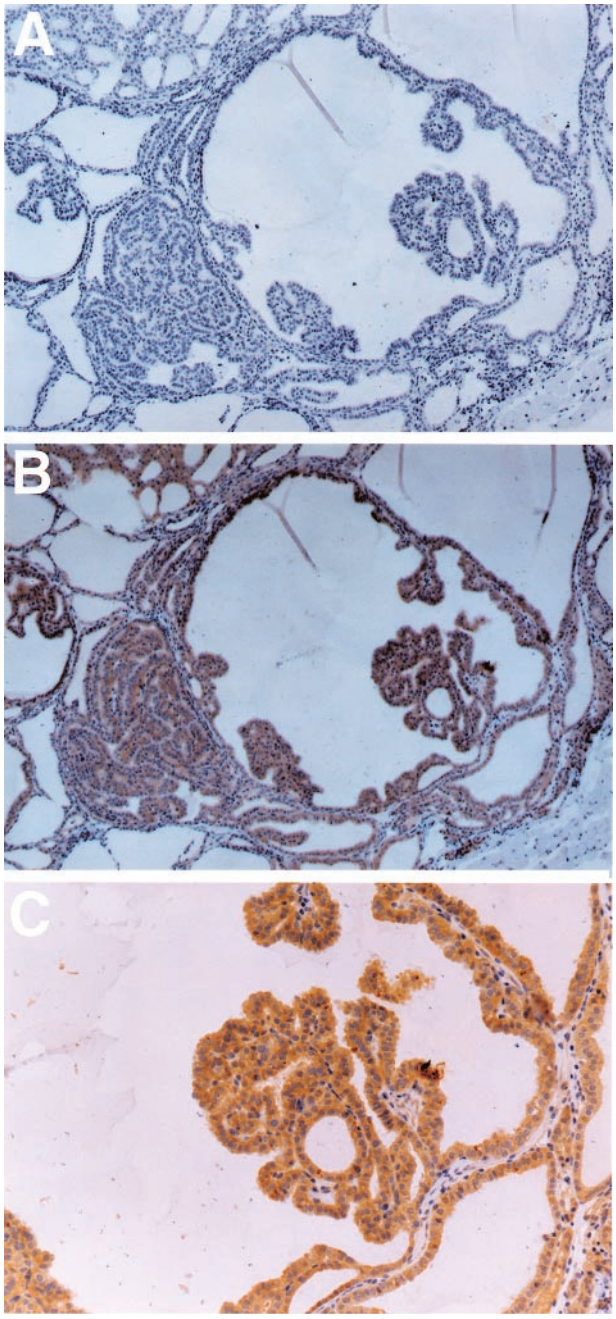

Figure 4 *TRK-T1* transgenic mice develop thyroid follicular hyperplasia. (**a**) Thyroid tissue from a *TRK-T1* mouse, line 5215, stained with secondary antibody alone as a negative control and counterstained with hematoxylin (magnification 10×). (**b**) Thyroid tissue from a *TRK-T1* transgenic mouse, line 5215, stained with an antibody specific to human NTRK1 and counterstained with hematoxylin. Thyroid follicular epithelial cells stain positively for human NTRK1 (magnification 10×) (**c**) Higher power magnification of specimen shown in (**b**) (magnification 20×)

for pathological changes of the thyroid according to the specific criteria outlined in Table 1. Although thyroids from the 5218 and 5221 *TRK-T1* transgenic lines showed similar histological changes, they were not included in Table 2. Overall, histological examinations of *TRK-T1* transgenic thyroids revealed a loss of normal thyroid architecture in 19/35 (54%) of thyroids examined. Interestingly, 16/26 or 62% of the thyroids from transgenic mice 7 months of age or younger were normal, while 100% of thyroids from mice older than 7 months of age showed thyroid abnormalities (Table 2). Examination of affected *TRK-T1* thyroids revealed increased follicular cellularity together with irregular or colloid-deficient follicles. This abnormal thyroid architecture was defined as hyperplasia and was absent from nontransgenic littermates (Table 1). In addition, analysis of these hyperplastic *TRK-T1* transgenic thyroids also revealed cellular invaginations into the follicle resembling micro-papillary structures often seen in differentiated human thyroid carcinoma (Figure 4). Hyperplasia was evident by 3 months of age, but onset was observed as late as 10 months. Furthermore, *TRK-T1* transgenic mice developed thyroid carcinoma characterized by the proliferation of follicular epithelial cells containing scant cytoplasm, absent mitotic figures and papillae containing fibrovascular stalks (Figure 5b), features characteristic of differentiated carcinomas in humans (Rosai *et al*., 1990). Thyroid carcinoma was

found in 6/26 or 23% or mice 7 months of age or younger (Table 2). In contrast, 7/9 or 78% of mice older than 7 months of age developed thyroid carcinoma. Solid sheets of cells resembling solid-type carcinoma were rare in tumor bearing *TRK-T1* transgenic thyroids.

Although human papillary thyroid carcinoma is characterized by distinct nuclear changes and lymphatic spread, we did not observe nuclear abnormalities or tumor metastases in local cervical lymph nodes, peripheral lymph nodes or lungs from *TRK-T1* mice. Finally, unlike the 33% of human PTC, which present with lymphocytic infiltrates (Rosai *et al*., 1990), tumors arising in the *TRK-T1* mice failed to induce inflammation. This observation however, likely reflects the state of immunological tolerance to unique antigens caused by *TRK-T1* gene expression early in development.

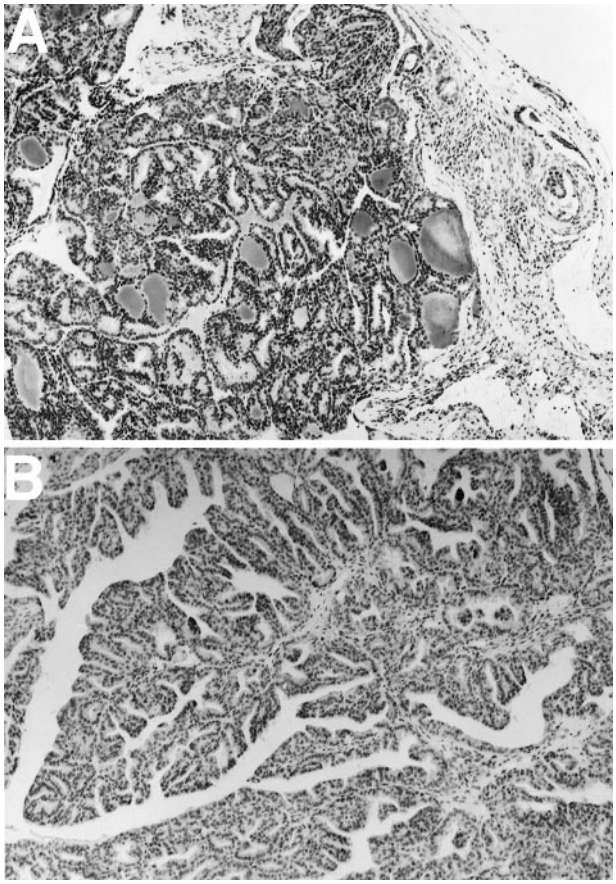

Figure 5 *TRK-T1* transgenic mouse thyroids synthesize thyroglobulin and develop carcinoma with characteristic papillary architecture. (**a**) A representative *TRK-T1*, line 5215, transgenic thyroid specimen stained positively with an antibody specific for murine thyroglobulin and counterstained with hematoxylin (magnification 10×). (**b**) A representative *TRK-T1* thyroid section, line 5215, stained with hematoxylin revealing papillae composed of fibrovascular stalks (magnification 10×)

Discussion

Two families of translocations involving the proto-oncogenes, *NTRK1* and *c-RET*, have been found in human papillary thyroid carcinoma (Bongarzone *et al*., 1989). The common feature of these families is the fusion of a ubiquitously expressed amino terminal partner to a carboxyl terminal partner containing a growth factor receptor tyrosine kinase domain resulting in the formation of an oncoprotein (Pierotti *et al*., 1996; Sozzi *et al*., 1992). These fusion proteins are thought to aberrantly activate mitogenic signaling pathways within the thyrocyte that result in increased growth and cell division (Wynford-Thomas, 1997). Moreover, thyroid hyperplasia may facilitate the accumulation of additional mutations that promote tumor progression and loss of a differentiated

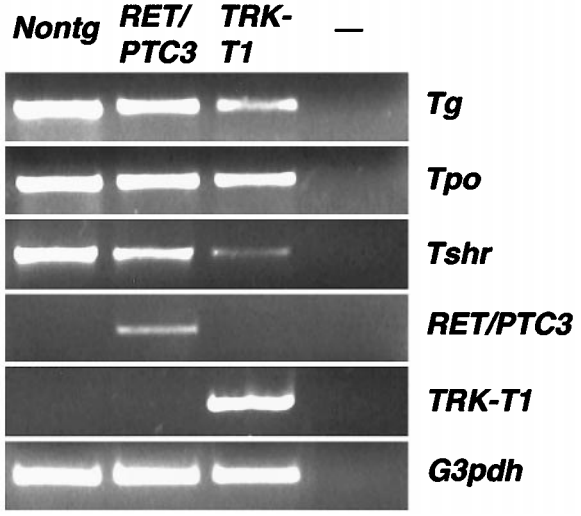

Figure 6 Semi-quantitative RT–PCR analysis for thyroglobulin, thyroid peroxidase and thyroid stimulating hormone receptor gene expression was performed on nontransgenic, *RET/PTC3* and *TRK-T1* transgenic mice. Total thyroid RNA from nontransgenic (Nontg), *RET/PTC3* and *TRK-T1* transgenic mice was isolated, reverse transcribed, and PCR amplified for the following genes: thyroglobulin (*Tg*), 350 bp; thyroid peroxidase (*Tpo*), 300 bp; thyroid stimulating hormone receptor (*Tshr*), 550 bp; *RET/PTC3* breakpoint (*RET/PTC3*), 310 bp; *TRK-T1* breakpoint (*TRK-T1*), 590 bp; and glyceraldehyde-3-phosphate dehydrogenase (*G3pdh*), 100 bp. PCR was performed after normalizing the amount of template cDNA with reference to *G3pdh* (see Materials and methods). Control PCR reactions performed without cDNA are indicated as (−). Control PCR reactions performed without reverse transcriptase were negative

Table 1 Criteria for pathological features described in *TRK-T1* transgenic mice

Thyroid	*Morphological description*
Normal	Thyroid specimens from wild type and non-transgenic mice have uniform colloid filled follicles with rare parafollicular cells. Follicles are composed of a one cell thick layer of cuboidal epithelium.
Hyperplasia	Increased numbers of thyroid follicular epithelial cells surrounding large (more than 2× normal) colloid deficient follicles. Cellular invagintions into the follicular lumen may also be present.
Carcinoma	More than 50 thyroid follicular epithelial cells clustered together with a homogenous appearance. Epithelial cell clusters may form a continuous sheet or papillae that surround a fibrovascular stalk. Nuclei may be either homogenous or pleiomorphic.
Metastatic	Evidence of thyroid cells outside the thyroid capsule and in regional lymph nodes or in other organ sites. Evidence of thyroid origin (e.g. *TRK-T1* or thyroglobulin gene expression).

Table 2 Pathological abnormalities observed in *TRK-T1*, thyroids, line 5215, based on age

*Age of mice at analysis**	*Thyroid pathology†*			*Total number of mice examined*
	Normal	*Hyperplasia*	*Carcinoma*	
⩽7 months	16	4	6	26
>7 months	0	2	7	9

*Mouse thyroid pathology followed a bimodal distribution that separated into two populations at 7 months of age. †See Table 1 for the description of pathological criteria used for these data

phenotype. However, analysis of human PTC has revealed variant translocations involving the proto-oncogene *NTRK1*, which appears less frequently than translocations involving *c-RET* (Beimfohr *et al*., 1999; Bongarzone *et al*., 1989, 1996, 1998a; Delvincourt *et al*., 1996; Fugazzola *et al*., 1995; Greco *et al*., 1992; Sozzi *et al*., 1992; Wajjwalku *et al*., 1992). The infrequent occurrence of *NTRK1* translocations and their inability to fully transform PC C13 *in vitro* warranted detailed analysis to measure the strength of their carcinogenic activity *in vivo*.

The pathology observed in *TRK-T1* transgenic mice demonstrates that fusion proteins containing the NTRK1 kinase are oncogenic in the mammalian thyroid. *TRK-T1* expression in the thyroid of transgenic mice induced thyroid epithelial cell transformation and hyperplasia that preceded the development of carcinoma indicating that *TRK-T1* is sufficient to initiate thyroid cancer *in vivo*. Moreover, the growth of papillae containing fibrovascular stalks in *TRK-T1* transgenic mouse thyroids, a histopathological feature observed in human cancer, supports this conclusion. Notably, *TRK-T1* carcinomas from all transgenic lines presented with the classic papillary structures, characteristics of human PTC, but infrequently with solid-type papillary carcinoma. In contrast, older *RET/PTC3* and *RET/PTC1* transgenic mice frequently developed solid-type carcinomas (Powell *et al*., 1998; Sagartz *et al*., 1997). Although, discrepancies between these models may be explained by subtle differences in the construct, mouse genetic background or undefined environmental factors, they are likely attributable to the function of the individual oncogenes since similar pathology is observed in thyroid tumors from both transgenic mice and humans expressing the *RET/PTC* fusion proteins (Powell *et al*., 1998).

TRK-T1 may be a weaker oncogene *in vivo* than *RET/PTC* since 46% of *TRK-T1* transgenic mice do not develop any detectable thyroid abnormalities, whereas thyroid carcinomas were observed in all *RET/PTC1* (Jhiang *et al*., 1996), and hyperplasia or carcinoma in all *RET/PTC3* transgenic mice (Powell *et al*., 1998). Moreover, a majority of *TRK-T1* transgenic mice presented with thyroid carcinoma only after 7 months of age and none of the mice developed poorly differentiated carcinoma through 24 months of observation. The incomplete penetrance of thyroid hyperplasia along with the lengthy time course for the development of thyroid carcinoma indicates that the *TRK-T1* fusion gene may allow thyroid follicular epithelial cells to survive and accumulate other genetic abnormalities that precipitate overt thyroid carcinoma. Consistent with this notion, transplanted *RET/PTC3* thyroid carcinomas fail to grow in normal or SCID mice whereas the poorly differentiated thyroid carcinomas transplanted from *RET/PTC*$^{p53-/-}$ mice grow progressively (Powell *et al*., submitted). Thus, *TRK-T1* mice crossed with other mice strains, such as those deficient in tumor suppressor genes or those expressing known oncogenes, may help uncover the pathways controlling thyroid tumor progression from indolent differentiated to transplantable poorly differentiated cancers.

The *TRK-T1* transgenic mouse strain provides a new animal model to study the various pathological differences evoked by *NTRK1* oncogene expression in thyroid epithelium. We find that the functional comparison between RET/PTC3 and TRK-T1 proteins has revealed differences in their capability to cause tumors in mice. Importantly, these results may reflect differences in the pathological outcome of papillary cancers observed in human disease. In addition, since these mouse strains reliably recapitulate human disease they provide an ideal system to examine the genetic and compensatory host changes during carcinogenesis. Furthermore, the characterization and study of these mice will aid in improving the diagnosis of human thyroid malignancies and in the testing of new anticancer therapies.

Materials and methods

Development of TRK-T1 transgenic mouse strain

A 5.4 kb DNA transgene was made from the bovine thyroglobulin promoter (2 kb) (Rochefort *et al*., 1996), a mammalian β-globin intron (0.7 kb), the human *TRK-T1* coding sequence (1.7 kb), and an SV40 polyadenylation signal (1 kb). The transgene was cloned into the pBluescript-II SK$^+$ vector (Stratagene, Inc., LaJolla, CA, USA) and plasmid DNA was purified using columns according to manufacturer's protocol (Qiagen, Inc., Santa Clarita, CA, USA). For transgenesis, 2 μg of purified construct DNA was microinjected into zygotes as described (Hogan *et al*., 1986). Founder animals were developed by the Kimmel Cancer Institute Transgenic Facility and identified by Southern hybridization using a *TRK-T1* specific probe (Ausubel *et al*., 1995). Founder mice were mated with wild type B6C3F1 and progeny screened for the presence of the transgene by PCR with transgene specific primers as described below.

DNA amplification by PCR

PCR was used to determine transgene presence using a modification of existing protocols (Ausubel *et al*., 1995). Briefly 100 ng of genomic DNA was added to a 50 μl reaction mixture containing 1× PCR buffer, 0.2 mM deoxynucleotide triphosphates, 50 pM 3′ and 5′ oligonucleotides (5′ primer sequence of *TRK-T1*, CACATCATCGAGAACCCACAA; and 3′ primer *TRK-T1*, GCTCATGCCAAAATCACCAAT which generate a 550 bp product) and 2.0 U Taq polymerase. The reaction tubes were then placed into a heated lid thermocycler (Hybaid, Inc.) and subjected to 30 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and elongation at 72°C for 60 s. PCR products were resolved on a 2% agarose gel and bands visualized with ethidium bromide staining and UV light illumination.

Thyroid cell transformation

The PC C13 thyroid epithelial cell line used in these studies was derived from 18-month-old Fischer rats (Fusco *et al*., 1987). PC C13 cells were maintained in Coon's modified F12

medium (GIBCO-BRL, Paisley, PA, USA) supplemented with 5% calf serum (GIBCO) and six hormones (6H; TSH, insulin, hydrocortisone, somatostain, transferrin and glycyl-histidyl-lysine) (Sigma Chemical, Co.), as described elsewhere (Fusco *et al*., 1987). 5×10^5 cells were plated 48 h before transfection in 60 mm tissue culture dishes. Three hours prior to transfection, the medium was changed to Dulbecco's modified Eagle's medium (DMEM) (GIBCO) containing 5% calf serum and 6H. Calcium phosphate precipitates of DNA (10 μg of *TRK-T1* or *NTRK1* (*pDM-69*) plasmid and 1 μg of *pSV2-neo*) were prepared as reported elsewhere (Fusco *et al*., 1987) and were incubated with the cells for 1 h. DNA precipitates were removed and cells were incubated in 15% glycerol for 2 min. Cells were washed with DMEM and incubated for 48 h in Coon's modified F12 medium supplemented with 5% calf serum and 6H. Transfectants were selected in 400 μg/ml Geneticin (G418, GibcoBRL). Two independent populations were obtained for the *TRK-T1* transfection and one for the NTRK1 transfection. Protein extractions and Western blot analysis (20 μg of total protein) were performed according to standard procedures. Immune complexes were detected with the enhanced chemiluminescence kit (Amersham Corp). The anti-TRK rabbit polyclonal antibody (c-14) was purchased from SantaCruz Biotechnology Inc. The NTRK1 expressing plasmid, *pDM-69*, used as a positive control was a gift from Dr M Barbacid, Bristol Myers Squibb.

RNAse protection analysis (RPA)

Thyroid tissue was removed from transgenic mice and homogenized in 0.5 ml of cell lysis buffer (4 M Guanidinium thiocyanate, 25 mM Sodium citrate, 0.5% Sodium N-lauroylsarcosine, 0.1 M 2-mercaptoethanol) using a mechanical homogenizer (Biospec Products, Racine, WI, USA). Protein was removed using phenol: chloroform extraction (1 : 1), while nucleic acid was recovered following ethanol precipitation. DNA was removed using RNAse free DNAse (Ambion). *TRK-T1* riboprobe was synthesized from a 180 bp cloned fragment (1054–1236) of the *TRK-T1* gene, while the control actin riboprobe was synthesized from a 330 bp vector (Ambion). Riboprobes and thyroid RNA were precipitated together, resuspended in 20 μl of hybridization buffer (80% deionized formamide, 100 mM sodium citrate, pH 6.4, 300 mM sodium acetate, pH 6.4, 1 mM EDTA) and incubated overnight at 42°C. RNA hybrids were digested with 40 μg/ml RNAse A and 2 μg/ml RNAse T1 for 30 min at 37°C. RNA was precipitated, resuspended in gel loading buffer (95% formamide, 0.025% xylene cyanol, 0.025% bromophenol blue, 0.5 M EDTA, 0.025% SDS), denatured for 3 min at 94°C and resolved on a 5% denaturing polyacrylamide gel for 1 h at 250 volts. Gels were dried and exposed to X-ray film.

Immunocytochemistry and pathological analysis

TRK-T1 transgenic mice between 2 and 24 months of age were sacrificed and thyroid, lung, cervical and peripheral lymph nodes (popliteal and para-aortic) were removed for pathological examination. Immunohistochemical analysis of protein expression in mouse tissues was performed using established laboratory protocols (Powell *et al*., 1998). Briefly, tissues were fixed in 10% formalin for 24 h and desiccated. Following fixation, tissue samples were embedded in liquid paraffin and cooled. Paraffin-embedded tissue was sliced into 6 μM sections and placed on silanized slides (Fisher Scientific). After deparaffinization in xylenes, the sections were hydrated through decreasing concentrations of alcohol and microwaved for 15 min in 100 mM citrate buffer (pH 6.0). Sections were blocked with 10% normal goat serum for 15 min and incubated with a rabbit polyclonal antibody against TRK A (1 : 500, TRK-763, Santa Cruz Biotechnology) or mouse monoclonal antibody against thyroglobulin (1 : 500, Harlan Sera-Lab, Loughborough, UK) overnight at room temperature. The following day, sections were washed twice for 5 min each with phosphate buffered saline (PBS) and once for 5 min with PBS/1% bovine serum. Sections were incubated with biotinylated secondary antibody for 1 h at room temperature, washed and incubated with substrate according to the DAB Vectastain kit (Vector Labs, Inc.), counterstained using hematoxylin, dehydrated and mounted. Thyroid sections were examined by a board-certified surgical pathologist (M Cunnane) and pathological criteria assessed.

Semi-quantitative RT–PCR analysis

Five μg total RNA was isolated as described above from *TRK-T1* transgenic and nontransgenic mouse thyroid, reverse transcribed (Superscript II, GibcoBRL) and cDNA amplified in a semi quantitative PCR after cDNA had been normalized using primers specific to glyceraldehyde-3-phosphate dehydrogenase (*G3pdh*). The murine *G3pdh* specific PCR primers were 5′ primer CCTTCATTGACCTCAACTAC and 3′ primer ATGACAAGCTTCCCATTCTC. Semi-quantitative PCR was then performed utilizing murine primers specific for thyroglobulin (5′ primer CCTGGTCTTGTGGGTCTCTA and 3′ primer GAGGAAGGTAGAGAGCATCG), thyroid peroxidase (5′ primer ATGAGAACACTTGGAGCTAT and 3′ primer GACTTGTATTGATGTTTCCA), thyroid stimulating hormone receptor (5′ primer GCAAAGAGTGTGCGTCTCCA and 3′ primer GCATCCAGCTTTGTTCCATT) and human primers specific for the *RET/PTC3* (5′ primer TGGAGAAGAGGAGCTGTATC and 3′ primer CTTTCAGCATCTTCACGG) and *TRK-T1* (5′ primer GCGGTGTTGCAGCAAGTCCT and 3′ primer CGATGATGTGGCCTTGGAGC) breakpoints as described above. The PCR product sizes generated were: *G3pdh*, 100 bp; thyroglobulin, 350 bp; thyroid peroxidase 300 bp; thyroid stimulating hormone receptor, 550 bp; *TRK-T1* breakpoint, 590; and *RET/PTC3* breakpoint, 310 bp. Control PCR reactions performed without cDNA did not produce any products. In addition, all RNA samples were sham reverse transcribed and PCR amplified to ensure that amplification products were derived from cDNA and not contaminating genomic DNA (-RT control).

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